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MEASUREMENTS OF GLYCOLYTIC INTERMEDIATES DURING THE ONSET OF THERMOGENESIS IN THE SPADIX OF *ARUM MACULATUM*

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SUMMARY

1. This work was done to compare the amounts of glycolytic intermediates in the club of the spadix of *Arum maculatum* L at an early stage (α) of development, immediately prior to the increase in glycolysis (pre-thermogenesis), and at the peak of the rapid glycolysis (thermogenesis).

2 Glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, 3-phosphoglycerate, 2-phosphoglycerate, phospho*enol*pyruvate and pyruvate were measured. The results indicate that at all the above stages of club development the reactions catalysed by phosphoglucomutase, glucosephosphate isomerase, phosphoglycerate mutase and enolase were close to equilibrium, but those catalysed by phosphofructokinase and pyruvate kinase were considerably displaced from equilibrium.

3. The amounts of the above compounds per club increased 5-fold between α stage and pre-thermogenesis but the relative amounts remained unchanged. When glycolysis increased by more than 50-fold at thermogenesis, the amount of fructose 1,6-diphosphate per club rose, but no changes were detected in the amounts per club of any of the other compounds listed above. These results are discussed in relation to the control of glycolysis.

INTRODUCTION

The thermogenic terminal portion, the club, of the spadix of *Arum maculatum* is a favourable tissue for the study of the control of carbohydrate oxidation in higher plants During flowering this club passes through a number of recognizable developmental stages In the early stages, called α and β, carbohydrate oxidation is via glycolysis and the pentose phosphate pathway. Subsequent development is accompanied by a shift towards glycolysis so that at pre-thermogenesis, the stage just prior to pollination, dependence upon glycolysis is almost complete. The next stage, called thermogenesis, is characterized by a massive and rapid rise in glycolysis that is accompanied by considerable heat production. In 4 h the rate of respiration may rise 70-fold and glycolysis may attain rates higher than that of tetanized muscle. During development from α stage to pre-thermogenesis the maximum catalytic activities of phospho-

fructokinase, aldolase, and glyceraldehyde-phosphate dehydrogenase rise markedly. These rises are a prerequisite for the increase in glycolysis at thermogenesis and are an example of coarse control of glycolysis. None the less the maximum catalytic activities of these enzymes do not increase between pre-thermogenesis and thermogenesis. Thus the immediate cause of the increase in the rate of glycolysis at thermogenesis must be some mechanism of fine control, and, or an increase in the availability of substrate [1–3]. The aim of the present work was to compare the amounts of glycolytic intermediates at α stage, pre-thermogenesis, and thermogenesis in order to determine the points at which glycolysis might be regulated.

The very considerable technical difficulties involved in measuring glycolytic intermediates in higher plants necessitates the provision of convincing evidence that such measurements represent the amounts of the compounds present in vivo [4]. We obtained this evidence as follows. For each test of the technique we prepared duplicate samples of tissue Each sample was freeze-clamped and dropped into separate amounts of cold $HClO_4$ for killing. For one of the samples, measured amounts of glycolytic intermediates were added to the $HClO_4$ with the freeze-clamped tissue. Comparison of the amounts of the intermediates recovered from the two samples is taken as a measure of the reliability of the complete process of killing, extraction, and measurement. We emphasize the following aspects of this procedure First is the need to study the effects of freeze-clamping on the amounts of the intermediates recovered from the tissue. Dropping plant tissue straight into liquid nitrogen introduces the possibility of changes in amounts of metabolites due to relatively slow cooling [5]. Second is the importance of carrying out recovery experiments by adding exogenous compounds to samples of tissue that has been effectively disrupted either by freeze-clamping or by homogenization in liquid nitrogen. This procedure means that as the ruptured tissue thaws in the $HClO_4$ the added compounds are much more accessible to enzymes in the tissue than is so when the exogenous compounds are added to tissue that is not homogenized until after killing. Finally it is important that the amounts of compounds added in the recovery experiment be comparable to the amounts present in the sample of tissue.

MATERIALS AND METHODS

Plants. All experiments were carried out with the swollen club-shaped portion of the appendix of the spadix of *A. maculatum* L. We used plants from a range of local natural sites. For experiments with clubs at α stage and at pre-thermogenesis, recognized as described previously [2], we collected whole inflorescences and within 90 min of collection we excised and immediately freeze-clamped the clubs. For measurements on clubs at thermogenesis, we dug up complete plants at the onset of thermogenesis, put them in a growth room at 14 °C and monitored the temperature of the club with a copper-constantan thermocouple as described by Rackham [6] The club temperature rose within the next 5 h, and when it reached its peak, the club was excised and freeze-clamped at once.

Analytical methods. For the straightforward measurement of glycolytic intermediates the samples of clubs taken for freeze-clamping contained: at α stage, 16–24 clubs (fresh weight, 3.7–6.0 g); at pre-thermogenesis, 9–13 clubs (fresh weight, 7.5–11 g); and at thermogenesis, single clubs (fresh weight, 0.6–1.5 g). For the recovery

experiments duplicate samples of the above size were used at α stage and pre-thermogenesis. At thermogenesis we took five clubs, split each longitudinally into halves, and placed the corresponding halves in different samples so that each final sample contained five half-clubs.

For freeze-clamping, up to 5 g fresh weight of tissue were put in a muslin bag and then rapidly and heavily clamped between two aluminium blocks (diameter, 7.5 cm; width, 3 cm) bolted onto the end of a pair of blacksmith's tongs. The blocks were pre-cooled to the temperature of liquid nitrogen. This clamping ruptured the tissue so completely that a club of 1 g was flattened to a thin disk 6 cm × 4 cm which produced a fine homogenate when allowed to thaw in solution. The freeze-clamped tissue was dropped at once into 3–4 volumes 1.41 M $HClO_4$ at 1 °C. The frozen mass which resulted was kept at −5 °C for 18 h. Then the suspension which had formed as the temperature rose to −5 °C was centrifuged at 34 000 × *g* for 4 min and the pellet was extracted twice more, each time with one volume of 1.41 M $HClO_4$ at −2 °C. All three extracts were combined, neutralized with 5 M K_2CO_3 and then centrifuged at 34 000 × *g* for 4 min. The sediment was washed thrice, each time with 1.41 M $HClO_4$ that had been neutralized with 5 M K_2CO_3. The washings, and the supernatants obtained from the extracts, were combined, shaken with 2–6 g activated charcoal, and the resulting suspension was filtered through glass fibre paper. The filtrate was taken for assay of the glycolytic intermediates by the spectrophotometric techniques described in the following references: glucose 1-phosphate [7], glucose 6-phosphate and fructose 6-phosphate [8], fructose 1,6-diphosphate [9], 3-phosphoglycerate, 2-phosphoglycerate, phospho*enol*pyruvate and pyruvate [10]. For measurement of pyruvate, the treatment with charcoal was omitted. In the recovery experiments measured amounts of glycolytic intermediates, roughly equal to the amounts present in the sample of tissue and dissolved in 1.0 ml water, were added to the $HClO_4$ with one of each pair of duplicate freeze-clamped samples. Fisher's *P* values were calculated by Student's *t*-test; values greater than 0.05 are regarded as not significant.

RESULTS AND DISCUSSION

Clubs at α stage and at pre-thermogenesis could be recognized reliably and consistently by their morphological characteristics [2]. Recognition of thermogenesis was more difficult because the maximum rate of glycolysis is maintained for only a short time and it is at this time that comparison with the other stages is best made. Simultaneous measurements of temperature and CO_2 production of clubs still attached to their plants have shown that throughout thermogenesis there is an extremely close correlation between these two properties of the club [3]. The clubs that we designate as thermogenic were freeze-clamped within minutes of reaching peak temperature. Thus glycolysis in these clubs must have been near its maximum rate when they were excised.

The reliability of our analyses may be assessed from the results of the recovery experiments (Table I). The recoveries indicate that our assays of glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate and phospho*enol*pyruvate at all three stages of development are satisfactory. The measurements for glucose 1-phosphate, 3-phosphoglycerate, 2-phosphoglycerate and pyruvate are less convincing but do not suggest massive losses of these compounds during killing and extraction. Further

TABLE I

RECOVERY OF GLYCOLYTIC INTERMEDIATES DURING KILLING AND EXTRACTION OF CLUBS OF SPADICES AT DIFFERENT STAGES OF DEVELOPMENT

In each experiment duplicate samples of clubs were prepared; one sample was freeze-clamped, added to $HClO_4$ and extracted, the other sample was treated similarly except that measured amounts of the intermediates were added to the $HClO_4$ with the sample. Both extracts were neutralized and assayed as described in the text. For each duplicate, the difference in the amounts of a compound found in the two extracts is expressed as a percentage of the amount added during killing, and is presented as an estimate of the recovery of the added compound. Values are means ±S E. from the number of experiments shown in parentheses

Compound	Estimate of recovery of added compound (%)		
	α stage	Pre-thermogenesis	Thermogenesis
Glucose 1-phosphate	77± 3 (3)	80±13 (4)	178
Glucose 6-phosphate	93± 6 (4)	88± 5 (4)	96± 3 (4)
Fructose 6-phosphate	81±16 (4)	73± 8 (4)	92±10 (4)
Fructose 1,6-diphosphate	110±20 (3)	90±18 (4)	103± 9 (4)
3-Phosphoglycerate	–	147, 108	105±16 (3)
2-Phosphoglycerate	–	96	180
Phospho*enol*pyruvate	107± 9 (3)	95± 6 (4)	137±31 (3)
Pyruvate	72±28 (3)	109, 75	105

evidence of the validity of the measurements of the latter compounds is provided by the fact that, in general, there was close agreement between clubs in our estimates of both the absolute (Table II) and relative (Table III) amounts present. The very small amounts of glucose 1-phosphate and 2-phosphoglycerate in the clubs made it very difficult to undertake recovery experiments with comparable amounts of these compounds. We could not obtain reliable measurements for dihydroxyacetone phosphate and phosphoglyceraldehyde. We found that treatment of the extracts with activated charcoal improved the recoveries of most of the compounds listed in Table I.

The amounts of the different glycolytic intermediates that we found in the clubs are given in Table II. These amounts are expressed per club because the number of cells in a club does not change much between α stage and thermogenesis, whereas fresh weight and protein content change appreciably [2]. We also weighed the samples of clubs that we analysed and we related the amounts of the glycolytic intermediates to the fresh weight of the clubs. These values are not given because they can be calculated from the data in Table II and previously published measurements of club weight [2] The fresh weight of the clubs used in the present experiments did not differ significantly ($P > 0.05$) from the previously published values. If our data are expressed per fresh weight, it can be seen that the amounts of glycolytic intermediates in the clubs, even at thermogenesis, are closely comparable to those reported as typical of meristematic tissue of plants [11]. From Table II it can be seen that for each compound there is some variation in the values that we obtained for comparable clubs. There is not enough published data to show whether such variation in estimates of glycolytic intermediates is a general feature of plant tissues. However, a similar degree of variation is characteristic of estimates from a wide range of animal tissues [12]. In general, the mass action ratios for the different glycolytic reactions show quite close agreement

TABLE II

AMOUNTS OF GLYCOLYTIC INTERMEDIATES IN CLUBS OF SPADICES AT DIFFERENT STAGES OF DEVELOPMENT

Samples of clubs were freeze-clamped, killed and extracted with $HClO_4$, and the extracts were analysed as described in the text Each value is the mean ±S E of estimates from the number of samples shown in parenthesis Fisher's *P* values are given for comparison of the amounts of intermediates in clubs at different stages of development Values of 0 05 or less are considered significant Values greater than 0 05 are given as n s (not significant)

Compound	Amount (nmol per club)			Fisher's *P* values		
	I α stage	II Pre-thermogenesis	III Thermogenesis	I vs II	II vs III	I vs III
Glucose 1-phosphate	4 9±0 2 (5)	19 7± 1 7 (5)	21 4± 3 3 (8)	< 0 001	n s	< 0 01
Glucose 6-phosphate	46 3±5 7 (5)	244 0±24 0 (5)	282 3± 42 0 (9)	< 0.001	n s	< 0 01
Fructose 6-phosphate	8 7±1 1 (5)	43 1± 4 9 (5)	52 6± 8 7 (9)	< 0 001	n s	< 0 01
Fructose 1,6-diphosphate	4 8±1 8 (4)	29 6± 6 1 (5)	92.9± 18 8 (8)	< 0 01	< 0 05	< 0 01
3-Phosphoglycerate	34 5±4 7 (3)	156 8±17 6 (5)	177 4± 37 0 (9)	< 0 01	n s	n s
2-Phosphoglycerate	2 9±0 5 (4)	15 2± 3 6 (4)	19 9± 3 2 (8)	< 0 02	n s	< 0 01
Phospho*enol*pyruvate	5 1±0 6 (5)	25 4± 1 2 (5)	41 0± 5 9 (8)	< 0 001	n s	< 0 001
Pyruvate	14 7±1 5 (5)	286 0±63 0 (5)	261 3±107 4 (4)	< 0 01	n s	< 0 05

TABLE III

COMPARISONS OF APPARENT EQUILIBRIUM CONSTANTS OF GLYCOLYTIC REACTIONS WITH RATIOS OF SUBSTRATES FOUND IN CLUBS AT DIFFERENT STAGES OF DEVELOPMENT

Apparent equilibrium constants are from Newsholme and Start [5] except that for phosphoglucomutase which is from Najjar [13] Substrates were measured as described in Table II Unless shown otherwise, ratios were calculated for each sample of tissue, and the values so obtained were averaged to give the figures presented which are means ±S E of the ratios from the number of samples shown in parentheses At pre-thermogenesis and thermogenesis, pyruvate and phospho*enol*pyruvate were not measured in the same samples of clubs, thus values marked * are ratios of the mean values for the two compounds from Table II

Reaction	Apparent equilibrium constant	Substrates	Ratios[a]		
			α stage	Pre-thermogenesis	Thermogenesis
Phosphoglucomutase	17 2	Glc-6-*P* Glc-1-*P*	9 45±1 18 (5)	12 55±0 97 (5)	13 64±1 46 (8)
Glucosephosphate isomerase	0 36–0 47	Fru-6-*P* Glc-6-*P*	0 19±0 01 (5)	0 18±0 01 (5)	0 18±0 01 (9)
Phosphofructokinase	900–1200	Fru-1,6-P_2· Fru-6-*P*	0 52±0 15 (4)	0 69±0 12 (5)	1 97±0 22 (8)
Phosphoglycerate mutase	0 1–0 2	2-*P*-Glyceric acid 3-*P*-Glyceric acid	0 10±0 04 (3)	0 10±0 01 (4)	0 14±0 03 (8)
Enolase	2 8–4 6	*P-enol*pyruvate· 2-*P*-Glyceric acid	1 77±0 37 (4)	1 87±0 40 (4)	2 47±0 52 (8)
Pyruvate kinase	2000–20 000	Pyruvate *P-enol*pyruvate	3 07±0 44 (5)	11 26*	6 37*

[a] For each individual reaction Fisher's *P* values were calculated for comparison of the ratios at the three stages of development Only two comparisons (Fru-1,6-P_2 Fru-6-*P*, pre-thermogenesis vs thermogenesis, $P < 0\ 01$, and α stage vs thermogenesis, $P < 0\ 01$) gave values of $P > 0\ 05$

between comparable clubs (Table III). Thus, although the absolute amounts of glycolytic intermediates may have varied between comparable clubs, the relative amounts were quite similar.

Comparison of the mass action ratios with the apparent equilibrium constants (Table III) provides strong evidence that at α stage, pre-thermogenesis, and thermogenesis, the reactions catalysed by phosphoglucomutase, glucosephosphate isomerase, phosphoglycerate mutase, and enolase were close to equilibrium. This indicates that none of these reactions plays a dominant role in the regulation of glycolysis during the development and thermogenesis of the club. Calculation of the mass action ratios for phosphofructokinase and pyruvate kinase requires knowledge of the relative amounts of ATP and ADP in the cytoplasmic or soluble phase of the cells. Techniques for the measurement of ATP and ADP in the cytoplasmic phase of non-photosynthetic plant cells have yet to be developed. Estimates of the total amounts of ATP and ADP in the club of the spadix of *Sauromatum guttatum*, a close relative of *A. maculatum*, indicate an overall ATP : ADP ratio of 0.6–1.6 that does not change significantly during the onset of thermogenesis [14]. We think that such measurements of total ATP and ADP should not be used to assess the equilibrium position of cytoplasmic enzymes in plant cells because there is considerable evidence from other eukaryotic cells that the relative amounts of ATP and ADP in the cytoplasm differ appreciably from those in the mitochondria [15]. Accordingly, we have used the lower values of the apparent equilibrium constants in Table III, and our estimates of the ratios fructose 1,6-diphosphate: fructose 6-phosphate, and pyruvate : phospho*enol*pyruvate to calculate the ATP : ADP ratios that would have to exist in the cytoplasm of the clubs if the phosphofructokinase and pyruvate kinase reactions were at equilibrium in vivo. For phosphofructokinase at α stage, pre-thermogenesis, and thermogenesis, these ratios would have to be $3.8 \cdot 10^{-4}$, $7.6 \cdot 10^{-4}$, and $2.5 \cdot 10^{-3}$, respectively The corresponding ratios for pyruvate kinase are 694, 178, and 314. All these values are so different from the ratios likely to exist in vivo that we suggest that at all three stages of development both the phosphofructokinase and pyruvate kinase reactions are considerably displaced from equilibrium, and are thus capable of acting as regulatory reactions.

A non-equilibrium reaction is established as regulatory if it can be shown that a change in flux is accompanied by a change in substrate concentration in the opposite direction. From Table II it can be seen that development of the club from α stage to pre-thermogenesis was accompanied by significant increases in the contents of all the intermediates assayed However, expression of our results on the basis of fresh weight, in the manner explained earlier, showed that there was no significant ($P > 0.05$) difference between the amounts of fructose 6-phosphate and phospho*enol*pyruvate per g fresh weight at α stage and the respective amounts at pre-thermogenesis. Further there were no significant differences between α stage and pre-thermogenesis in respect of the mass action ratios shown in Table III. Comparison of pre-thermogenic and thermogenic clubs shows that we found no significant change in the contents per club of any of the intermediates measured except for the increase in fructose 1,6-diphosphate. The only ratio that we found to alter was that of fructose 1,6-diphosphate : fructose 6-phosphate ($P < 0\ 01$). This change was due to the rise in fructose 1,6-diphosphate and not to any fall in fructose 6-phosphate

The outstanding feature of our results is that we did not detect any evidence of a fall in the contents of fructose 6-phosphate or phospho*enol*pyruvate when glycolysis in

the club accelerated rapidly, and by as much as 50-fold, at thermogenesis. We checked that the amounts of the glycolytic intermediates in thermogenic clubs did not alter in the few minutes which elapsed between excision of the club and freeze-clamping. We did this by freeze-clamping clubs that were still attached to their plants. The values obtained in this way did not differ significantly from those in Table II. Further there is ample evidence that excised clubs exhibit the abnormally high rates of glycolysis [1, 2]. Examination of clubs at the onset and during the respiratory rise at thermogenesis provided no evidence of transient changes in the amounts of fructose 6-phosphate and phospho*enol*pyruvate. We also found no evidence for any fall at thermogenesis in glucose 1-phosphate or glucose 6-phosphate, compounds apparently in equilibrium with fructose 6-phosphate, or in 3-phosphoglycerate and 2-phosphoglycerate, compounds which appear to be in equilibrium with phospho*enol*pyruvate. The above arguments, and the recoveries shown in Table I, provide strong evidence that the marked increase in glycolysis at thermogenesis is not accompanied by any pronounced fall in the amounts of fructose 6-phosphate and phospho*enol*pyruvate, although both compounds are substrates of non-equilibrium reactions.

The club of *A. maculatum* appears to be exceptional but not unique in respect of the behaviour of fructose 6-phosphate and phospho*enol*pyruvate when glycolysis is increased. In a wide range of animal tissues changes in glycolytic flux are accompanied by opposite changes in fructose 6-phosphate [5]. However, the electric organ of *Electrophorus* [16], and frog sartorius muscle [17] are exceptions. Falls in phospho*enol*pyruvate when glycolysis is increased have been demonstrated in some but not all of the animal tissues examined [5]. The situation in higher plants is more difficult to assess because of the relative lack of authenticated measurements of glycolytic intermediates. There is evidence that increased glycolysis in some higher plant tissues was accompanied by falls in both fructose 6-phosphate and phospho*enol*pyruvate [18–21] whereas in other instances change in fructose 6-phosphate was small or undetectable although phospho*enol*pyruvate fell appreciably [22, 23]. Failure to observe a fall in the substrate of a non-equilibrium reaction when flux through the reaction is increased does not prove that the reaction is not regulatory [24]. Our data for *Arum* may reflect a limitation of glycolysis by the availability of hexose phosphate, or a situation in which hexose phosphate production is stimulated to a greater degree than phosphofructokinase. The increase in fructose 1,6-diphosphate that accompanies stimulation of glycolysis in *Arum* is difficult to interpret. It is, however, important to bear in mind that changes in fructose 1,6-diphosphate may not be used in conjunction with either the cross-over theorem or mass action ratios to prove that the phosphofructokinase reaction is regulatory [24].

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